Rapid Identification of Dengue Virus by Reverse Transcription-Polymerase Chain Reaction Using Field-Deployable Instrumentation

Guarantor: James C. McAvin, MS
Contributors: James C. McAvin, MS*; Elizabeth M. Escamilla, MS*; Maj Jamie A. Blow, USAF†; Michael J. Turell, PhD†; Miguel Quintana, PhD‡; Maj David E. Bowles, USAF§; Col James A. Swaby, USAF§; Maj William J. Barnes, USAF*; Col William B. Huff, USAF*; Kenton L. Lohman, PhD*; Maj Daniel H. Atchley, USAF*; Lt Col John R. Hickman, USAF*; Lt Col Debra M. Niemeyer, USAF‖¶

Dengue virus universal and dengue serotype 1 to 4, fluorogenic probe hydrolysis (TaqMan), reverse transcription-polymerase chain reaction assays were developed for screening and serotype identification of infected mosquito vectors and human sera using a field-deployable, fluorometric thermocycler. Dengue universal and dengue 1 to 4 serotype assay in vitro sensitivity and specificity results were 100% concordant when tested with total nucleic acid extracts of multiple strains of dengue serotype 1 to 4, yellow fever, Japanese encephalitis, West Nile, and St. Louis encephalitis viruses. The in vitro sensitivity and specificity results for all five assays were concordant when tested with a blind panel of 27 dengue virus-infected mosquitoes, 21 non-dengue (yellow fever, West Nile, or St. Louis encephalitis) flavivirus-infected mosquitoes, and 11 uninfected mosquitoes and with clinical specimens consisting of a human serum panel of eight dengue viremic and 31 non-dengue-infected febrile patient serum samples. No cross-reaction occurred with vector species or human genomic DNA. Sample processing and polymerase chain reaction required <2 hours.

Introduction

Dengue fever (DF) and the more severe forms of the disease, i.e., dengue hemorrhagic fever (DHF) and dengue shock syndrome, occur in tropical and subtropical regions globally through infection by one or more of four viral serotypes (dengue serotypes 1–4).[1] Dengue virus is a member of the genus *Flavivirus*, family Flaviviridae, that is transmitted in a cycle that primarily involves humans and mosquito vectors, most significantly *Aedes aegypti* in developing urban regions and *A. aegypti* as well as *Aedes albopictus* in semiurban areas and rural regions.[1–5] The prevalence of dengue virus is now comparable to that of malaria, making DF the most significant mosquito-borne viral disease, threatening two-fifths of the world's human population.[6,7] It is estimated that 50 to 100 million people are affected annually with DF and 300,000 with DHF/dengue shock syndrome.[6]

Dengue virus surveillance and DF/DHF diagnoses are problematic. Symptoms are usually nonspecific, and serological analyses or virus isolation can take 1 week or more.[1,8,9] Antibody cross-reaction occurs across the Flaviviridae family, creating ambiguity.[10,11] To augment clinical laboratory diagnostic capability, dengue virus reverse transcription (RT)-polymerase chain reaction (PCR) assays have been developed for use with laboratory-based instrumentation.[12–18] In areas in which clinical laboratory diagnostics are not available, field surveillance is an essential element in achieving timely assessments of transmission risk and time-critical implementation of appropriate mosquito control measures and clinical responses in a potential outbreak situation. Rapid identification of circulating virus serotypes and causative serotypes in previous outbreaks is fundamental to risk assessment for DHF.

Given the expansion of expeditionary operations worldwide, U.S. Armed Forces are at risk for acquiring a variety of infections, including dengue. After World War II, a dengue pandemic in Southeast Asia marked expanded emergence of the virus, with epidemics caused by multiple serotypes.[16] With no licensed vaccine available, DF and other febrile diseases remain a threat to U.S. military operations, as observed throughout the history of conflict[19] and most recently during Operation Restore Hope.[20] This threat drives the need for improved far-forward surveillance and rapid diagnosis of febrile illnesses. In the past several years, PCR has been shown to be a military field-worthy technique for rapid identification of biological agents.[21–23] In this work, we describe a PCR-based assay system for rapid, sensitive, specific screening of dengue virus and serotype identification in mosquito vectors and human sera, using deployable instrumentation.

*Epidemiological Surveillance Division, U.S. Air Force Institute for Environment, Safety, and Occupational Health Analysis, Brooks Air Force Base (now designated Air Force Institute for Operational Health, Brooks City-Base), San Antonio, TX 78235-5237.

†Virology Division, U.S. Army Medical Research Institute for Infectious Disease, Fort Detrick, Frederick, MD 21702-5011.

‡Environmental Science Division, U.S. Army Center for Health Promotion and Preventative Medicine-West, Fort Lewis, WA 98433-9500.

§U.S. Air Force Force Protection Battlelab, Lackland Air Force Base, San Antonio, TX 78236-0119.

‖U.S. Air Force Office of the Surgeon General, Bolling Air Force Base, Washington, DC, 20332-0103.

¶Joint Program Executive Office for Chemical and Biological Defense, Falls Church, VA 22041-3203.

This manuscript was cleared through the Technical Publication/Presentation Control Record, Brooks City-Base, TX for open publication, January 2004. The conclusions and opinions expressed in this document are those of the authors. They do not reflect the official position of the U.S. government, Department of Defense, Joint Program Office for Chemical and Biological Defense, U.S. Air Force, U.S. Army, or the Association of Military Surgeons of the United States.

Mention of a trade name, vendor, proprietary product, or specific equipment is not an endorsement, a guarantee, or a warranty by the Department of Defense, Army, or Air Force and does not imply an approval to the exclusion of other products or vendors that also may be suitable.

This manuscript was received for review in January 2004. The revised manuscript was accepted for publication in December 2004.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 DEC 2005	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Rapid identification of dengue virus by reverse transcription-polymerase chain reaction using field-deployable instrumentation, Military Medicine 170:1053 - 1059	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
McAvin, JC Escamilla, EM Blow, JA Turell, MJ Quintana, M Bowles, DE Swaby, JA Barnes, WJ Huff, WB Lohman, KL Atchley, DH Hickman, JR Niemeyer, DM	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Dengue virus universal and dengue seroype 1 to 4, fluorogenick probe hydrolysis (Taqman) reverse transcription-polymerase chain reaction assays were developed for screening and serotype identification of infected mosquito vectors and human sera using a field-deployable, fluorometric thermocycler. Dengue universal and dengue 1 to 4 serotype assay in vitro sensitivity and specificity results were 100% concordant when tested with nucleic acid extracts of multiple strains of dengue serotype 1 to 4, yellow fever, Japanese encephalitis, West Nile, and St. Louis encephalitis viruses. The in vitro sensitivity and specificity results for all five assays were concordant when tested with a blind panel of 27 dengue virus-infected mosquitoes, 21 non-dengue (yellow fever, West Nile, or St. Louis encephalitis) flavivirus-infected mosquitoes and 11 uninfected mosquitoes and with clinical specimens consisting of a human serum panel of eight dengue viremic and 31 non-dengue-infected febrile patient serum samples. No cross-reaction occurred with vector species or human genomic DNA. Sample processing and polymerase chain reaction required < 2 hours.

15. SUBJECT TERMS

dengue, rapid identification, PCR, reverse trancription, field deployable

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**8**	

Methods

Primer and Probe Design

Given that an established, TaqMan-based, (Synthetic Genetics, Rockville, Maryland), RT-PCR, universal dengue virus assay was not obtainable from the literature, primer and probe sequences were selected by aligning homologous genomic regions of serotypes 1 to 4 that excluded other clinically significant flaviviruses. Alignments were compared visually using the Clustal algorithm[24] in the MegAlign program of DNA Star software (Perkin Elmer, Norwalk, Connecticut).[25] Maximally conserved oligonucleotide sequences were chosen from dengue virus type 1 to 4 genomes downloaded from GenBank (accession numbers U88536, M19197, M93130, and AF326825, respectively). Yellow fever, Japanese encephalitis, West Nile, and St. Louis encephalitis virus-type strain genomic sequences were aligned and visually evaluated to validate heterology with universal primer and probe sequences (GenBank accession numbers X03700/K02749, M18370, M12294/M10103, and AF242895, respectively). Universal primer and probe sequences can be requested through the corresponding author. Serotype-specific primer and probe sequences were obtained from the literature (DEN-1,[12] DV-2,[13] DEN-3,[12] and DEN-4[12]), and heterology was validated as described above. All probes reported here are dual fluorogenic labels designed with a 5′ reporter dye, 6-carboxyfluorescein, and a 3′ quencher dye, 6-carboxytetramethylrhodamine.[26]

Universal and serotype-specific primers and probes sequence heterology with genomic sequences of closely related species through diverse genera was validated by BLAST database search (BLAST, Madison, Wisconsin).[27] Melting temperatures were quantified and the absence of significant primer dimerizations and secondary structure (hairpin) formations was confirmed with PrimerExpress software (PE Applied Biosystems, Foster City, California). Primers and probes were synthesized and quality control was conducted commercially (Synthetic Genetics, San Diego, California). Assays were designated DU-JCM, DEN-1, DEN-2, DEN-3, and DEN-4, respectively.

Flavivirus Reference Strains

To optimize RT-PCR assay sensitivity and specificity, nucleic acid extracts of reference strains of dengue virus serotypes and other flaviviruses were obtained from the Southwest Foundation for Biomedical Research (San Antonio, Texas) and the Center for Tropical Diseases, University of Texas Health Science Center (Galveston, Texas). Species and strain nucleic acid extracts included dengue 1 (Hawaii, Mochizuki, and 106), dengue 2 (S16803, NGC, VEN2, 131, Mara-3, Mex44, IQ2913, and 102954), dengue 3 (CH53489, H87, and 53402), dengue 4 (H241, Dakar NC599, and VEN4), yellow fever (Asibe, TVP834, and TVP8249), Japanese encephalitis (JKT9092, PhAn1091, and JKT8442), West Nile virus (Crow 397–99, TVP3545, and TVP8345), and St. Louis encephalitis (Fort Washington, TVP8314, TVP8344, and LA2001).

Assay Optimization

Relative sensitivity levels of each assay were optimized with six logarithmic dilutions of each dengue serotype, prepared by diluting nucleic acid extracts 1:10 in RT-PCR-grade water with 2 U of RNase inhibitor. The specificity of each assay was tested with undiluted samples and four logarithmic dilutions of each heterologous dengue serotype and other flaviviruses, prepared by dilution as described above.

Mosquito Panels

To validate RT-PCR assay in vitro sensitivity and specificity, mosquito panels were prepared at the Virology Division, U.S. Army Medical Research Institute for Infectious Disease (USAMRIID) (Fort Detrick, Maryland). One- to 6-day-old, adult, female, *A. aegypti* mosquitoes were inoculated intrathoracically[28] with one of the following viruses: dengue 1 (Hawaii), dengue 2 (S16803), dengue 3 (CH53489), dengue 4 (H241), yellow fever (Asibe), West Nile (Crow 397–99), or St. Louis encephalitis (Fort Washington). Mosquitoes were held in cardboard cages, provided with a carbohydrate source (either apple slices or a gauze pad soaked in a 10% sucrose solution) and a water-soaked cotton pledget, and held at 26°C for 7 days. Mosquitoes were then killed by exposure to −20°C for 5 to 10 minutes, and one to six legs were removed for analyses. Legs were triturated in grinding diluent (10% heat-inactivated fetal bovine serum in medium 199 with Earle's salts, $NaHCO_3$, and antibiotics) and tested for the presence of virus by plaque assay on LLC-MK-2 cells. One virus-inoculated or one unexposed mosquito body was added to each pool of uninfected mosquitoes. These were placed in sterile, 1.5-mL, Eppendorf tubes and triturated in 750 μL of TRIzol-LS (Life Technologies, Rockville, Maryland). A panel was established, labeled under code at USAMRIID, and shipped on dry ice to the Epidemiological Surveillance Division, U.S. Air Force Institute for Environment, Safety, and Occupational Health Analysis, Brooks Air Force Base (San Antonio, Texas), for nucleic acid extraction and blind RT-PCR analyses (Table I).

Clinical Specimens

Human blood specimens were collected during a DF epidemic in Tegucigalpa, Honduras, in February 2002, by the U.S. Army Center for Health Promotion and Preventative Medicine-West in collaboration with the Honduran Ministry of Health (HMOH), from a total of 39 febrile and convalescent patients 0 to 5 days after the onset of fever and after defervescence, respectively. The HMOH confirmed the presence of dengue virus by serological testing (Table II), and the specimens were archived at −20°C. Sera were subsequently thawed, and 500-μL aliquots of each specimen were prepared and labeled under code by the HMOH and shipped on dry ice to the Air Force Institute for Environment, Safety, and Occupational Health Analysis. Serum samples were stored at −20°C for 2 weeks, thawed, maintained at 4°C for 72 hours during nucleic acid extractions and blind validation testing with RT-PCR assays, and archived at −20°C. Before RT-PCR analyses, each serum sample had undergone at least two freeze-thaw cycles. The duration of exposure of the specimens to ambient temperatures throughout collection, processing, serological analyses, and preparation for shipping is unknown.

RNA Preparation

Individual mosquitoes and mosquito pools were homogenized in 500 μL and 1,000 μL of TRIzol (Life Technologies), respectively, with sterile, RNase-free pestles and 1.5-mL tubes, ho-

TABLE I

RT-PCR RESULTS OF DENGUE VIRUS ASSAY TESTING OF INFECTED MOSQUITOES

Panel	Virus	Strain	Plaque-Forming Units per Leg	DU-JCM	DEN-1	DEN-2	DEN-3	DEN-4
101	Dengue 1	Hawaii	3	Positive	Positive	—	—	—
104	Dengue 4	H241	3.6	Positive	—	—	—	Positive
105	None		1 uninfected	—	—	—	—	—
106	Dengue 1	Hawaii	3.2	Positive	Positive	—	—	—
109	Dengue 4	H241	3.6	Positive	—	—	—	Positive
110	None		1 uninfected	Positive	—	—	—	—
112	Dengue 4	H241	3	Positive	—	—	—	Positive
113	Dengue 1	Hawaii	2.9	Positive	Positive	—	—	—
114	None		1 uninfected	Positive	Positive	—	—	—
117	Dengue 4	H241	2.6	Positive	—	—	—	Positive
118	None		1 uninfected	—	—	—	—	—
120	Dengue 1	Hawaii	3	Positive	Positive	—	—	—
121	None		1 uninfected	—	—	—	—	—
123	Dengue 4	H241	3.3	Positive	—	—	—	Positive
124	Dengue 1	Hawaii	3	Positive	Positive	—	—	—
125	None		1 uninfected	—	—	—	—	—
127	Dengue 4	H241	3.1	Positive	—	—	—	Positive
128	Dengue 1	Hawaii	3	Positive	Positive	—	—	—
401	Dengue 3A	H87	2	Positive	—	—	Positive	—
402	Dengue 2	S16803	3.9	Positive	—	Positive	—	—
403	Dengue 3B	CH53489	3.6	Positive	—	—	Positive	—
404	None		1 uninfected	—	—	—	—	—
405	Dengue 2	S16803	4.2	Positive	—	Positive	—	—
406	Dengue 3A	H87	3.6	Positive	—	—	Positive	—
407	Dengue 2	S16803	3.6	Positive	—	Positive	—	—
408	Dengue 3B	CH53489	4	Positive	—	—	Positive	—
409	None		1 uninfected	—	—	—	—	—
410	None		1 uninfected	—	—	—	—	—
411	Dengue 3A	H87	2.8	Positive	—	—	Positive	—
412	Dengue 3B	CH53489	4	Positive	—	—	Positive	—
413	Dengue 2	S16803	4.3	Positive	—	Positive	Positive	—
414	None		1 uninfected	—	—	—	—	—
415	Dengue 2	S16803	4.3	Positive	—	Positive	—	—
416	Dengue 3B	CH53489	3.9	Positive	—	—	Positive	—
417	Dengue 3A	H87	2.2	Positive	—	—	Positive	—
418	None		1 uninfected	—	—	—	—	—
419	Dengue 3B	CH53489	3	Positive	—	—	Positive	—
420	Dengue 3A	H87	3	Positive	—	—	Positive	—
201	Yellow fever	Asibe	4	—	—	—	—	—
202	St. Louis encephalitis	Fort Washington	5	—	—	—	—	—
203	West Nile	Crow 397–99	>5	—	—	—	—	—
204	Diluant		0	—	—	—	—	—
205	Yellow fever	Asibe	3.9	—	—	—	—	—
206	St. Louis encephalitis	Fort Washington	5	—	—	—	—	—
207	West Nile	Crow 397–99	>5	—	—	—	—	—
208	Diluant		0	—	—	—	—	—
209	West Nile	Crow 397–99	>4.5	—	—	—	—	—
210	Diluant		0	—	—	—	—	—
211	Yellow fever	Asibe	3.7	—	—	—	—	—
212	St. Louis encephalitis	Fort Washington	>4.0	—	—	—	—	—
213	West Nile	Crow 397–99	>4.5	—	—	—	—	—
214	St. Louis encephalitis	Fort Washington	>4.0	—	—	—	—	—
215	West Nile	Crow 397–99	>4.5	—	—	—	—	—
216	Diluant		0	—	—	—	—	—
217	Yellow fever	Asibe	4	—	—	—	—	—
218	St. Louis encephalitis	Fort Washington	>4.0	—	—	—	—	—
219	Yellow fever	Asibe	3.1	—	—	—	—	—
220	Diluant		0	—	—	—	—	—
221	West Nile	Crow 397–99	>4.5	—	—	—	—	—
222	St. Louis encephalitis	Fort Washington	>4.0	—	—	—	—	—
223	Yellow fever	Asibe	4	—	—	—	—	—
224	Diluant		0	—	—	—	—	—
225	West Nile	Crow 397–99	>4.5	—	—	—	—	—
226	Yellow fever	Asibe	4	—	—	—	—	—
227	St. Louis encephalitis	Fort Washington	>4.0	—	—	—	—	—
228	Diluant		0	—	—	—	—	—

TABLE II

RT-PCR RESULTS OF DENGUE VIRUS ASSAY TESTING OF HUMAN SERUM

No.	Sample	Collection Date	Serological Test	DU-JCM	DEN-1	DEN-2	DEN-3	DEN-4
1	02-2935	7/7/2002	—	—	—	—	—	—
2	02-2938	7/7/2002	—	—	—	—	—	—
3	02-2945	5/7/2002	—	—	—	—	—	—
4	02-2948	7/7/2002	—	—	—	—	—	—
5	02-2943	6/7/2002	—	—	—	—	—	—
6	02-2964	3/7/2002	—	—	—	—	—	—
7	02-2965	4/7/2002	—	—	—	—	—	—
8	02-2958	7/7/2002	—	—	—	—	—	—
9	02-2963	3/7/2002	Dengue 2	Positive	—	Positive	—	—
10	02-2968	3/7/2002	—	—	—	—	—	—
11	02-2934	6/7/2002	—	—	—	—	—	—
12	02-2955	7/7/2002	Dengue 2	Positive	—	Positive	—	—
13	02-2957	6/7/2002	Dengue 2	Positive	—	Positive	—	—
14	02-2960	3/7/2002	—	—	—	—	—	—
15	02-2932	6/7/2002	—	—	—	—	—	—
16	02-2931	7/7/2002	—	—	—	—	—	—
17	02-2933	5/7/2002	—	—	—	—	—	—
18	02-2942	7/7/2002	—	—	—	—	—	—
19	02-2921	5/7/2002	—	—	—	—	—	—
20	02-2888	3/7/2002	—	—	—	—	—	—
21	02-2882	5/7/2002	Dengue 2	Positive	—	Positive	—	—
22	02-2878	4/7/2002	—	—	—	—	—	—
23	02-2972	3/7/2002	—	—	—	—	—	—
24	02-2909	2/7/2002	—	—	—	—	—	—
25	02-2978	08/07/02	—	—	—	—	—	—
26	02-2117	27/06/02	—	—	—	—	—	—
27	02-2115	27/06/02	Dengue 2	Positive	—	Positive	—	—
28	02-1720	23/06/02	—	—	—	—	—	—
29	02-2238	1/7/2002	Dengue 2	Positive	—	Positive	—	—
30	02-2969	3/7/2002	—	—	—	—	—	—
31	02-2144	30/06/02	Dengue 2	Positive	—	Positive	—	—
32	02-2114	29/06/02	—	—	—	—	—	—
33	02-2101	26/06/02	Dengue 2	Positive	—	—	—	—
34	02-2125	1/7/2002	—	—	—	—	—	—
35	02-1846	25/06/02	—	—	—	—	—	—
36	02-2874	4/7/2002	—	—	—	—	—	—
37	02-2116	27/06/02	—	—	—	—	—	—
38	02-2877	4/7/2002	—	—	—	—	—	—
39	02-2869	4/7/2002	—	—	—	—	—	—

All patient samples were collected during the acute febrile phase.

mogenates were cleared by centrifugation in a tabletop centrifuge at 12,000 rpm for 60 seconds, and total nucleic acid extracts were prepared according to the manufacturer's instructions. Each extract was suspended in 100 μL of RNase-free, PCR grade water with 2 U of RNase inhibitor.

Human serum specimens were thawed at 4°C, and 140 μL of each specimen were suspended in 560 μL of the buffer AVL/carrier RNA component of a preformatted, total nucleic acid extraction kit (QIAamp viral RNA mini kit, Qiagen, Valencia, California). Extracts were prepared and suspended in 60 μL of elution buffer with the manufacturer's centrifugation protocol.

Reaction Conditions

Preliminary assay optimizations and cross-reaction testing were conducted with a laboratory-based, real-time thermocycler, the LightCycler (Idaho Technology, Salt Lake City, Utah),[29] with preformatted reagents (Titan one-tube RT-PCR kit, Roche Molecular Biochemicals, Indianapolis, Indiana). Assays were reoptimized for the Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.) (Idaho Technology) with a proprietary buffer system (Idaho Technology), and sensitivity and specificity validation testing was completed.

Master mixture reaction solution was prepared, 18-μL volumes were dispensed into optical capillary tubes, and 2 μL of RNA extract from specimens and control samples were added (or 2 μL of PCR-grade water for no-template control samples). Capillaries were placed in a tabletop centrifuge and centrifuged for 10 seconds at 3,000 rpm, to drive the assay mixture to the bottom of the tube. Master mixture components were 0.20 mM concentrations of dATP, dTTP, dGTP, and dCTP (Idaho Technology), 2.5 mM Mn(AOc) (Roche Molecular Biochemicals), 2.5 U of Tth polymerase (Roche Molecular Biochemicals), and 20% (v/v)

proprietary 5× RT buffer (Idaho Technology). The dengue virus universal assay (DU-JCM) forward primer concentration was 0.50 μM, reverse primer 0.50 μM, and TaqMan probe 0.50 μM. DEN-1, DEN-2, DEN-3, and DEN-4 had the same primer and probe concentrations; the forward primer concentration was 0.50 μM, reverse primer 0.90 μM, and TaqMan probe 0.10 μM.

A standardized RT-PCR thermal cycling protocol was established, consisting of RT at 40°C for 30 minutes followed by initial cDNA denaturation at 94°C for 2 minutes and PCR for 45 cycles of 94°C for 0 seconds for template denaturation and 60°C for 20 seconds for combined annealing and primer extension. A single data point at the end of each annealing-extension cycle was collected and reported as TaqMan probe fluorescence released by 5′-nuclease activity during primer extension. Fluorimeter gains were set at 8, 2, and 2 for channels 1, 2, and 3, respectively. Protocols for all five assays were identical with the exception of DEN-3, for which the extension temperature was 66°C. The criterion for a positive result was a significant increase in fluorescence over background levels, as defined by an algorithm provided in the R.A.P.I.D analytical software (Roche Molecular Biochemicals).

Results

Assay Sensitivity and Specificity Testing with Flavivirus Reference Strains

Preliminary assay sensitivity and specificity evaluations were conducted with a known panel of total nucleic acid extracts from dengue serotype 1- to 4-infected *A. aegypti* inoculated with three different strains of dengue 1, eight strains of dengue 2, three strains of dengue 3, and three strains of dengue 4 and a cross-reactivity test panel consisting of total nucleic acid extracts from multiple strains of other Flaviviridae (three strains each of yellow fever, Japanese encephalitis, and West Nile and four strains of St. Louis encephalitis). In vitro sensitivity and specificity of each assay were 100% concordant. No cross-reactivity was observed with vector species (data not shown).

Assay Sensitivity and Specificity Testing with Mosquito Panels

Testing of assay in vitro sensitivity and specificity was accomplished with a blind panel of 27 dengue-infected mosquitoes (six dengue 1-infected mosquitoes, five dengue 2, 10 dengue 3, and six dengue 4), 21 non-dengue-infected mosquitoes (seven yellow fever virus-infected *A. aegypti* and seven each of West Nile and St. Louis encephalitis virus-infected *Culex* spp.), and 11 uninfected mosquitoes, with 27 positive control samples and 32 negative control samples (Table I). Diluent samples were not included in statistical analyses. In the case of serotype-specific assay analyses, heterologous serotypes were considered as negative specimens. Assay results were as follows: DU-JCM, sensitivity of 100% (27 of 27 samples) and specificity of 94% (30 of 32 samples); DEN-1, sensitivity of 100% (six of six samples) and specificity of 98% (52 of 53 samples); DEN-2, sensitivity of 100% (five of five samples) and specificity of 100% (54 of 54 samples); DEN-3, sensitivity of 100% (10 of 10 samples) and specificity of 98% (48 of 49 samples); DEN-4, sensitivity of 100% (six of six samples) and specificity of 100% (53 of 53 samples). The DU-JCM assay reported two false-positive results (panels 110 and 114). Dengue 1 and 3 assays reported one false-positive result each (panels 114 and 413, respectively) (Table I). The finding that both DU-JCM and DEN-1 assays reported panel 114 as dengue virus positive indicates experimental error. Because one occurrence of a false-positive result was observed when the DEN-3 assay was tested against dengue 2 (panel 413), additional testing will be performed to further delineate observed results. No cross-reaction was observed with vector species genomic DNA and medium diluent. Sample processing and RT-PCR required <2 hours.

Assay Sensitivity and Specificity Testing with Clinical Specimens

Testing was accomplished with a blind panel of eight dengue viremic (dengue 2) and 31 non-dengue-infected febrile patient serum specimens (Table II). Dengue virus universal assay in vitro sensitivity was 100% (eight of eight samples) and specificity was 100% (31 of 31 samples). DEN-2 assay in vitro sensitivity was 88% (seven of eight samples) when tested against the human serum panel, with one false-negative result, and specificity was 100% (31 of 31 samples) when tested against non-dengue-infected specimens. Dengue 1, 3, and 4 assays displayed no cross-reactivity with dengue 2 serotype. Nucleic acid integrity of the human serum samples presented an unknown variable because of a lack of history regarding collection conditions, transportation and storage temperatures, and number of freeze-thaw cycles. Human genomic DNA displayed no detectable fluorescence above background levels. Sample processing and real-time RT-PCR required <2 hours.

Discussion

We have described a rapid, sensitive, specific dengue virus RT-PCR assay system for screening and serotype identification of infected mosquito vectors and human sera. The augmentation of traditional laboratory-based testing with field-deployable RT-PCR can enhance dengue virus surveillance capability and provides a promising aid in rapid clinical laboratory diagnostics in fixed-site and deployed medical locales.

Development of pathogen identification assays with field-durable instrumentation fulfills a fundamental requirement in the realization of a complete field-deployable assay platform. Although the R.A.P.I.D provides field-deployable, real-time PCR instrumentation, an omnipotent, field-deployable, assay platform requires additional components, including thermostable assay reagents and control nucleic acid, and field-formatted sample preservation and nucleic acid isolation technologies. Therefore, in 2001, the Department of Defense established the Joint Biological Agent Identification and Diagnostic System program to develop a complete field-ready system for rapid medical surveillance and diagnostic testing at fixed-site and deployed locations.[30] The system and components (protocols and assays) will be cleared by the Food and Drug Administration for patient care use. Our experiences described below emphasize the need for a standardized system approach.

Surveillance studies conducted under austere conditions present logistical and operational constraints that make it impractical to transport, store, and prepare PCR reagents and control nucleic acid. Where resources for cold chain mainte-

nance are unreliable or nonexistent, unknown variables in enzymatic activity and control nucleic acid integrity result. Moreover, sample integrity comes into question when sample collections are made distant to the laboratory or support facilities. Exposure to ambient temperature and freeze-thaw occurrences during transportation often present unknown variables in nucleic acid integrity of the sample. Also, nucleic acid extraction and preservation reagents designated as hazardous materials present logistical constraints because of transportation and shipping restrictions.

To meet logistical and operational constraints, we have focused most intensely on the most vulnerable components of the field-deployable assay platform, namely, PCR enzymes and control nucleic acid template. The inherent fragility of RNA makes maintaining RT-PCR target template integrity an especially daunting task. To ensure the validity of field-formatted assays, we are integrating thermostable, hydrolytic enzyme-shielded, Armored RNA control template (Ambion RNA Diagnostics, Austin, Texas) and lyophilized master mixture reagents (Idaho Technology) into our research protocols. These products provide master mixture reagents and positive control samples that are easily transported, are field-sustainable, and require only hydration and addition of sample template for analyses. In addition to the value of Armored RNA in meeting logistical and operational constraints, there is also promise for development of quantitative viral assays.[31] Our preliminary laboratory evaluation of commercial, preformatted, thermostable, nucleic acid isolation reagents has shown promise for improved field-collected sample processing as well as sample stabilization, particularly for RNA-based viruses. Therefore, the lessons learned from this study and subsequent studies, along with probe information and protocols, will be provided to the Joint Program Executive Office for Chemical and Biological Defense, as stipulated in the Critical Reagents Program multilaboratory agreement. Under this agreement, information and materials provided by government laboratories are evaluated for use in development of assays, protocols, and procedures for joint efforts, including the Joint Biological Agent Identification and Diagnostic System program (unpublished data).

In summary, this article illustrates the potential operational utility of using dengue virus assays for rapid, sensitive, and specific screening and serotype identification in mosquito vectors and human sera, on field-deployable instrumentation. This system also shows promise for development into a fully deployable package for epidemiological surveillance of dengue virus and a field-expedient method to augment traditional clinical laboratory diagnostic techniques.

Acknowledgments

Thanks go to Dr. Philip Armstrong, Southwest Foundation for Biomedical Research (San Antonio, Texas), and Dr. Robert Tesh, Center for Tropical Diseases, University of Texas Health Science Center (Galveston, Texas), for providing flavivirus nucleic acid extracts.

References

1. Gubler DJ: Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 1998; 11: 480–96.
2. Black WC, Bennett KE, Gorrochotegui-Escalante N, et al: Flavivirus susceptibility in *Aedes aegypti*. Arch Med Res 2002; 33: 379–88.
3. Kow CY, Koon LL, Yin PF: Detection of dengue viruses in field caught male *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) in Singapore by type-specific PCR. J Med Entomol 2001; 38: 475–9.
4. Rosen L: Dengue hemorrhagic fever. Bull Soc Pathol Exot 1996; 89: 91–4.
5. Tu WC, Chen CC, Hou RF: Ultrastructural studies on the reproductive system of male *Aedes aegypti* (Diptera: Culicidae) infected with dengue 2 virus. J Med Entomol 1998; 35: 71–6.
6. Centers for Disease Control and Prevention: Dengue fever. Available at http://www.cdc.gov/ncidod/dvbid/dengue/index.htm; accessed November 4, 2003.
7. World Health Organization: Dengue and dengue haemorrhagic fever: fact sheet 117. Available at http://www.who.int/mediacentre/factsheets/fs117/en/index.html; accessed November 4, 2003.
8. Gibbons RV, Vaughn DW: Dengue: an escalating problem. BMJ 2002; 324: 1563–6.
9. Hayes EB, Gubler DJ: Dengue and dengue hemorrhagic fever. Pediatr Infect Dis J 1992; 11: 311–7.
10. Schwartz E, Mileguir F, Grossman Z, Mendelson E: Evaluation of ELISA-based sero-diagnosis of dengue fever in travelers. J Clin Virol 2000; 19: 169–73.
11. Koraka P, Zellar H, Niedrig M, Osterhaus AD, Groen J: Reactivity of serum samples from patients with a flavivirus infection measured by immunofluorescence assay and ELISA. Microbes Infect 2002; 4: 1209–15.
12. Callahan JD, Wu SJ, Dion-Schultz A, et al: Development and evaluation of serotype- and group-specific fluorogenic reverse-transcriptase PCR (TaqMan) assays for dengue virus. J Clin Microbiol 2001; 39: 4119–24.
13. Houng HH, Hritz D, Kanesa-thasan N: Quantitative detection of dengue 2 virus using fluorogenic RT-PCR based on 3′-noncoding sequence. J Virol Methods 2000; 86: 1–11.
14. Houng HH, Chung-Ming Chen R, Vaughn DW, Kanesa-thasan N: Development of a fluorogenic RT-PCR system for quantitative identification of dengue virus serotypes 1–4 using conserved and serotype-specific 3′ noncoding sequences. J Virol Methods 2001; 95: 19–32.
15. Laue T, Emmerich P, Schmitz H: Detection of dengue virus RNA in patients after primary or secondary dengue infection by using the TaqMan automated amplification system. J Clin Microbiol 1999; 37: 2543–7.
16. Warrilow D, Northill JA, Pyke A, Smith GA: Single rapid TaqMan fluorogenic probe based PCR assay that detects all four dengue serotypes. J Med Virol 2002; 66: 524–8.
17. Sudiro TM, Ishiko H, Green S, et al: Rapid diagnosis of dengue viremia by reverse transcriptase-polymerase chain reaction using 3′-noncoding region universal primers. Am J Trop Med Hyg 1997; 56: 424–9.
18. Drosten C, Gottig S, Schilling S, et al: Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse-transcriptase-PCR. J Clin Microbiol 2002; 40: 2323–30.
19. Institute of Medicine: Strategies to Protect the Health of Deployed U.S. Forces: Medical Surveillance, Record Keeping, and Risk Reduction, pp 21–32. Washington, DC, Institute of Medicine, 1999. Available at http://www.nap.edu/openbook/0309066379/html/21-30.htm; accessed January 2, 2004.
20. Sharp TW, Wallace MR, Hayes CG, et al: Dengue fever in U.S. troops during Operation Restore Hope, Somalia, 1992–1993. Am J Trop Med Hyg 1995; 53: 89–94.
21. Niemeyer D, Jaffe R, Wiggins L: Feasibility determination for use of polymerase chain reaction in the U.S. Air Force Air-Transportable Hospital field environment: lessons learned. Milit Med 2000; 165: 816–20.
22. Niemeyer D: Rapid Biological Agent Identification Kenny Class Initiative After Initiative Report. Lackland Air Force Base, TX, Air Force Force Protection Battlelab, 2000.
23. MacDonald R, Cao T, Borschel R: Multiplexing for detection of multiple biological warfare agents shows promise in the field. Milit Med 2001; 166: 237–9.
24. Thompson JD, Higgins DG, Gibson TJ, Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994; 1: 4673–80.
25. Clewley JP, Megalign AC: The multiple alignment module of Lasergene. Methods Mol Biol 1997; 70: 119–29.
26. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP: Continuous fluorescence monitoring of rapid cycle DNA amplification. BioTechniques 1997; 22: 130–8.

27. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ: Basic local alignment search tool. J Mol Biol 1990; 15: 403–10.
28. Rosen L, Gubler D: The use of mosquitoes to detect and propagate dengue viruses. Am J Trop Med Hyg 1974; 23: 1153–60.
29. Wittwer CT, Ririe KM, Andre RV, David DA, Gundry RA, Balis UJ: The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. BioTechniques 1997; 2: 176–81.
30. Department of the Army: Joint Biological Agent Identification and Diagnostic System presolicitation notice. Federal Business Opportunities Solicitation DASG60-02-R-0008. Available at http://www.eps.gov/servlet/Solicitation/R/USA/SMDC/DASG60/DASG60-02-R-0008.
31. Pasloske BL, Walkerpeach CR, Obermoeller RD, Winkler M, DuBois DB: Armored RNA technology for production of ribonuclease resistant viral RNA controls and standards. J Clin Microbiol 1998; 36: 3590–4.